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The apolipoprotein B concentration in gingival crevicular fluid increases in patients with diabetes mellitus

Emiko Noguchi [a,b], Rina Kato [b], Kayoko Ohno [c], Atsushi Mitsui [b], Takashi Obama [b], Tsutomu Hirano [c], Hiroyuki Itabe [b,*], Matsuo Yamamoto [a]

[a] Department of Periodontology, Showa University School of Dentistry, Kitasenzoku, Ota-ku, Tokyo 145-8515, Japan
[b] Division of Biological Chemistry, Department of Molecular Biology, Showa University School of Pharmacy, Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan
[c] Division of Diabetes, Metabolism and Endocrinology, Department of Medicine, Showa University School of Medicine, Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

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ABSTRACT

Objective: Oral health conditions have a significant relationship with diabetes mellitus (DM) as well as dyslipidemia. In this study, we investigated the levels of apolipoprotein B (apoB) and oxidized low-density lipoprotein (oxLDL) in the gingival crevicular fluid (GCF) from patients with DM.

Methods: GCF and blood samples from 18 DM patients and 18 healthy subjects were examined. GCF was collected with paper points without inflicting any harm. The apoB and oxLDL levels were measured by sandwich ELISA assays.

Results: The number of teeth with a deep probing pocket depth and the number of teeth with bleeding on probing, two typical periodontal parameters, correlated with the DM parameters, such as hemoglobin A1c. The GCF volume and the concentrations of protein, apoB and oxLDL in GCF were significantly higher in the DM patients than in the healthy subjects. In particular, the apoB concentration in GCF was increased 6-fold in the DM patients. The GCF apoB concentration correlated well with the DM parameters in plasma.

Conclusion: GCF could be a clinical source for examining not only the oral status of patients, but also certain systemic conditions.

Introduction

Gingival crevicular fluid (GCF) is the physiological exudate present in the crevice between a tooth and the surrounding gingival tissues. Although the source of GCF has not been fully established [1], GCF contains various plasma proteins and components derived from local gingival cells [2,3]. As periodontal disease progresses, the volume of GCF increases and inflammatory cytokines appear in the GCF. GCF can be easily and non-invasively collected using dental paper points, so that it has been studied as a potential target for clinical examinations [4].

It is becoming increasingly evident that oral health conditions have relevance to systemic diseases. In particular, periodontal disease is considered to be one of the complications of diabetes mellitus (DM) [5]. Periodontal disease is caused by a chronic infection in which the periodontal tissues are eroded by both the pathogens present in the periodontal pockets and the inflammatory responses to them, such that the alveolar bone is absorbed and ultimately a loss of teeth occurs [6]. It is well established that patients with DM have at least a 2-fold increase in the risk of periodontal disease compared with non-diabetic subjects [7]. Furthermore, unlike most of the other diabetic complications, periodontal disease and DM have a reciprocal interaction [8], namely, periodontal disease is a risk factor for DM, while DM patients are more susceptible to periodontal disease.

It is well known that patients with DM often exhibit an increased risk for cardiovascular disease due to some combination of hyperglycemia, hyperlipidemia, obesity and hypertension in a given patient [9,10]. Epidemiological studies have reported an association between periodontal disease and dyslipidemic conditions [11]. The presence of *Porphyromonas gingivalis*, one of the major pathogenic bacteria of periodontal disease, has been suggested in atherosclerotic plaques, using PCR analysis to detect the specific DNA fragments [12]. Extensive treatment of patients with periodontal disease is

Abbreviations: apoB, apolipoprotein B; BMI, body mass index; BOP, bleeding on probing; CM, chylomicron; DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; GA, glycoalbumin; GCF, gingival crevicular fluid; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; mAb, monoclonal antibody; MDA-LDL, malondialdehyde-modified low-density lipoprotein; NGSP, National Glycohemoglobin Standardization Program; oxLDL, oxidized low-density lipoprotein; pAb, polyclonal antibody; PPD, probing pocket depth; ROC, receiver operating characteristics; TG, triacylglycerol; VLDL, very low-density lipoprotein.

* Corresponding author at: Division of Biological Chemistry, Department of Molecular Biology, Showa University School of Pharmacy, 1-5-8 Hatanodai, Shinagawa, Tokyo 142-8555, Japan. Fax: +81 3 3784 8219.
E-mail address: h-itabe@pharm.showa-u.ac.jp (H. Itabe).

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associated with an improvement in endothelial function [13]. However, whether periodontal disease is a direct cause of atherosclerotic vascular diseases has yet to be proven conclusively [14].

Based on these backgrounds, we thought that GCF is potentially a good source to investigate the relationship between systemic diseases and oral conditions. Previously we showed that apolipoprotein B (apoB) is present in GCF and that oxidized low-density lipoprotein (oxLDL) is enriched even in the GCF from healthy subjects [15]. In this study, we examined the amounts of apoB and oxLDL in the GCF collected from DM patients and healthy subjects. The GCF was sampled from the gingival tissues unafflicted with periodontal disease to investigate whether GCF changes under the conditions of a systemic disease such as DM.

Materials and methods

Patients

The subjects enrolled in this study were fully informed of the protocol of this study and written informed consent was obtained according to the Helsinki Declaration. The experimental protocol for this study was approved by the ethical committee of Showa University. A total of 40 subjects (22 patients and 18 control subjects) were recruited from July 2011 to July 2012. Those patients who had been diagnosed as having type 1 or 2 DM (HbA1c > 7.8%; NGSP values) for more than one year before the study were hospitalized for approximately one week as an inpatient education program for diabetes. The smoking history was checked according to a standardized questionnaire. Subjects requiring periodontal surgery were excluded. Additional exclusion criteria included a history of periodontal therapy, the current use of antibiotics or anti-inflammatory drugs within the previous 3 months, pregnancy or lactation, and fewer than 20 teeth. Ultimately, 18 patients with DM hospitalized in Showa University Hospital and 18 healthy subjects were examined. The basic characteristics, including age, gender, and duration of DM, are summarized in Table 1. Among the 18 DM patients, only one patient was type 1. Two GCF samples at the sites of two different teeth and a venous blood sample were collected from each participant.

The periodontal status and the parameters for hyperglycemia and hyperlipidemia

All of the DM patients and healthy subjects received a dental examination. For examination of periodontal status the probing pocket depth (PPD) and bleeding on probing (BOP) at the deepest site of each tooth were recorded. The degree of periodontal disease is evaluated by the periodontal scores, which are the average PPD of all the teeth, the percentage of pocket sites with PPD more than 4 mm and the percentage of BOP-positive sites. Dental plaque was collected from the two teeth used for GCF sampling. Total bacterial counts were calculated by real-time PCR using DNA probes specific for 5 major bacteria causing periodontal disease at the BML Co. (Tokyo, Japan).

Venous blood (26 mL) was drawn from a brachial vein using a collecting tube, and 21 mL out of the 26 mL blood was used to measure the following parameters: HbA1c was measured using a latex coagulation method, while glycoalbumin (GA), fasting blood glucose, triglyceride (TG), LDL-cholesterol (LDL-C), and HDL-cholesterol (HDL-C) were measured using enzymatic methods, and malondialdehyde-modified LDL (MDA-LDL) was measured using an ELISA procedure, all of which were performed at the BML Co. The rest of the sample was immediately centrifuged at 15,000 $\times g$ for 10 min to separate the plasma for the determination of the apoB and oxLDL concentrations. The subjects' diet was not modified in any way prior to blood collection. The chylomicron (CM), very low-density lipoprotein (VLDL) and LDL fractions were separated using sequential ultracentrifugation, as described previously [16]. Protein concentrations were determined using BCA protein assay reagent with BSA as the standard. The CM, VLDL and LDL samples were stored at 4 °C until analysis.

Table 1
Clinical characteristics of the subjects.

	Non-diabetes n = 18	Diabetes n = 18	*p*-Value
Gender (male/female)	13/5	12/6	0.7
Age (years)	50.2 ± 6.4	59.1 ± 15.1	0.029*
BMI (kg/m^2)	22.1 ± 3.4	24.2 ± 5.4	0.159
HbA1c (NGSP %)	5.4 ± 0.5	11.4 ± 2.5	0.0001*
Glycoalbumin (GA %)	14.1 ± 2.0	30.3 ± 7.9	0.0001*
Fasting blood glucose (mg/dL)	88.3 ± 25.2	202.8 ± 63.4	0.0001*
Duration of diabetes (month)	0	109.5 ± 105.5	
Smoker/non-smoker	1/17	4/14	0.14
LDL-C (mg/dL)	120.1 ± 23.9	145.7 ± 52.3	0.075
HDL-C (mg/dL)	57.7 ± 16.4	43.9 ± 8.1	0.004*
TG (mg/dL)	133.2 ± 68.4	187.7 ± 72.6	0.027*
MDA-LDL (U/L)	99.6 ± 41.7	131.2 ± 64.5	0.145

Values are given as mean ± SD.
* *p*-Value for comparisons between the non-DM and DM subjects (Welch test) was <0.05.

Collection of the GCF samples

GCF samples were collected using a method described previously [15]. Briefly, two buccal maxillary anterior teeth with shallow pockets were selected in each subject. When the average PPD of the two sites was more than 4 mm, the subject was dropped from this study to minimize the effects of local gingival inflammation on GCF. The site of GCF collection was isolated with cotton wool rolls, and saliva was gently removed from the adjacent tooth surface and gingiva using a blower brush. Two standard paper points (DENTSPLY International Inc., York, PA, USA) were gently inserted in the buccal gingival crevice for 1 min and then transferred into 100 μL of ice-cold sterilized phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Sigma-Aldrich, product no. P2714; final 5% v/v). After 5 min, the same procedure was repeated with new paper points, and the volume of GCF was measured using a pre-calibrated Periotron 8000 (ProFlow Inc., New York, NY, USA). GCF samples were stored at 4 °C until use. In total, 35 GCF samples for each group were examined, since we were able to recover only one GCF sample from one DM patient and one control subject.

Determination of the apoB and oxLDL concentrations

The concentrations of oxLDL in the GCF and plasma samples were measured using a sandwich ELISA protocol [15,16] that was modified slightly to improve the sensitivity. Briefly, 96-well microtiter plates (Costar, product no. 3369; NY, USA) were incubated with 100 μL of the anti-oxidized phospholipid mAb DLH3 (diluted to 10 μg/mL in PBS) [17] at RT with shaking for 2 h and then at 4 °C for four days. After blocking the plates with TBS containing 2% BSA, 100 μL of samples was incubated in the wells at 37 °C for 2 h and then at 4 °C overnight. The plates were subsequently washed four times with 340 μL of TBS containing 0.1% Tween-20 (TBS-T). The plates were incubated with a sheep anti-human apoB pAb (Binding Site Inc., Birmingham, UK) at RT with shaking for 2 h, then washed four times with TBS-T. A biotin-conjugated donkey anti-sheep pAb (Millipore; 1:50,000 diluted in TBS containing 1% skimmed milk) was added to the wells and incubated at 37 °C for 1 h. After washing four times with TBS-T, a complex of streptavidin and biotinylated alkaline phosphatase (Dako Cytomation; 1:100 diluted in Tris–HCl, pH 7.8) was added to the wells and incubated at 37 °C for 1 h. The plates were washed four times with TBS-T and then once with 100 μL of 50 mM Tris–HCl, pH 8.8. The plates were incubated with 100 μL per well of *p*-nitrophenyl phosphate solution for 1 h at 37 °C. Copper induced-oxidized human LDL was used as the standard. Under the present assay conditions 0.05 ng protein/well of oxLDL was detected, which is approximately one order more sensitive than the original procedures. The measurement of apoB was carried out by

sandwich ELISA as described previously [15]. The apoB values in the LDL, VLDL and CM fractions were combined to estimate the total apoB plasma concentration.

Statistical analysis

The results are presented as the means ± standard deviation. For the statistical analysis, Welch t-test and Pearson's correlation were carried out using the Statistical Package for the Social Sciences (version 18.0, SPSS Inc., Chicago, IL, USA) software. A p value < 0.05 was taken to be significant.

Results

Basic characterization of the DM patients

Table 1 shows the characteristics of the DM patients (n = 18) and non-DM subjects (n = 18) examined in this study. The levels of HbA1c, GA and fasting blood glucose clearly indicated poor glycemic control in the DM patients. The patients had been diagnosed with DM for 109.5 months on average (3–360 months). Body mass index (BMI), the smoker/non-smoker ratio, LDL-C and MDA-LDL were all slightly higher in the DM patients, but not significantly. HDL-C was lower and TG was higher in the DM patients, suggesting an association of lipid metabolism with DM.

Periodontal parameters of the DM patients

Periodontal disease is reportedly the sixth most common complication of DM. As periodontal disease progresses, the connective tissue surrounding the teeth is destructed and the alveolar bone absorbed, the probing pocket depth (PPD) correspondingly increases. In terms of the clinical criteria, PPD ≥ 4 mm is defined as periodontitis. GCF samples were collected from two non-diseased teeth per subject; the average PPDs of the GCF collection sites were less than 3 mm (Table 2). Bleeding on probing (BOP) indicates the disease activity at the probing site. The percentages of teeth with deep PPD or BOP and the number of residual teeth were significantly higher in the DM patients than non-DM subjects (Table 2). The total bacterial counts for the collection sites were not significantly different between the two groups.

As shown in Table 3, the periodontal scores correlate with the DM parameters. BOP correlates positively with HbA1c, GA and fasting blood glucose. Even stronger correlations are observed between the percentage of deep PPD and the DM parameters. The percentage of deep PPD also correlates with TG and MDA-LDL.

Table 2
Periodontal characteristics of the subjects.

	Non-diabetes n = 18	Diabetes n = 18	p-Value
Full-mouth measurements			
Probing pocket depth (PPD)			
Percentage of diseased sites (PPD ≥ 4 mm)	5.1 ± 7.8	24.5 ± 15.4	0.0001*
Bleeding on probing (BOP)			
Percentage of bleeding sites	3.2 ± 3.9	15.5 ± 11.3	0.001*
Number of residual teeth	27.7 ± 2.6	24.2 ± 3.5	0.0002*
Collection sites			
Average PPD of collection sites (mm)	2.1 ± 0.3	2.8 ± 0.7	0.0001*
Total bacterial count (10^4 copy)	1.1 ± 1.3	2.1 ± 2.3	0.2

Values are given as mean ± SD.
Full-mouth measurements of probing pocket depth (PPD) and bleeding on probing (BOP) were recorded using a manual probe.
* p-Value for comparisons between the non-DM and DM subjects (Welch test) was <0.05.

Table 3
Correlations between the periodontal parameters and the hyperglycemic and hyperlipidemic parameters in plasma.

	HbA1c (NGSP)	GA	Fasting blood glucose	TG	LDL-C	MDA-LDL
Percentage of diseased site (PPD ≥ 4 mm)	.518*	.572*	.461*	.274**	.201	.244**
Percentage of bleeding sites (BOP)	.367**	.243**	.298**	.176	.024	.108
Total bacterial count	.223	.220	.246**	.024	.201	.040

* Correlations significant at $p < 0.01$ level by Pearson's correlation test.
** Correlations significant at $p < 0.05$ level by Pearson's correlation test.

The GCF from DM patients and healthy subjects shows different characteristics

GCF is easily and non-invasively collected using dental paper points. The volume and protein concentration of the collected GCF samples were significantly higher in the DM patients than in the non-DM subjects ($p < 0.01$) (Fig. 1a, b). In addition, attention was focused on certain components of GCF, since we had identified apoB and oxLDL in GCF in a previous study [15]. The apoB and oxLDL concentrations in GCF, as determined by sandwich ELISA procedures, were significantly higher in the DM than non-DM subjects ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 1c, d). The GCF apoB concentration was significantly higher in the DM patients even when the data was adjusted for age (DM 653.2 ± 939.6 ng/mL vs. non-DM 82.9 ± 42.7 ng/mL; $p < 0.01$).

Table 4 shows the relationship between GCF and the plasma parameters. The GCF volume positively correlated with the DM parameters. The GCF protein concentration also positively correlated with GA and fasting blood glucose. However, the GCF volume and GCF protein concentration did not correlate with the hyperlipidemic parameters of TG, LDL or MDA-LDL. We found that the apoB in GCF showed a good correlation with the DM parameters, and it correlated significantly with TG, LDL and MDA-LDL, respectively. On the other hand, the apoB and oxLDL in GCF did not display any correlation with either apoB or oxLDL in plasma.

Fig. 2 shows the receiver operating characteristic (ROC) analysis of the apoB and oxLDL concentrations in GCF conducted for the purpose of DM prediction. The curve for the apoB concentration in GCF runs very close to the upper left corner, and the area under the curve in GCF apoB was 0.90, which is statistically significant ($p < 0.01$). Given that the GCF samples were collected from non-diseased sites, this data indicates that 80% of the DM patients could be predicted specifically by apoB in the GCF collected from healthy teeth without any false positives under an appropriate threshold. An effective cut-off value was 138 ng/mL, by which the data shows 86% sensitivity and 94% specificity. The curve for the oxLDL concentration in GCF partly overlaps the diagonal line, indicating that it is not suitable for the prediction of DM.

Discussion

GCF is the physiological exudate in the crevices between teeth and the surrounding gingiva. Since GCF can be collected easily without any substantial harm, it is potentially useful for clinical examinations. We found previously that apoB and oxLDL are present in the GCF taken from subjects who were healthy both periodontally and systemically [15]. In the current study, we found the apoB concentrations to be remarkably higher in the GCF from the DM patients than the healthy subjects. In particular, the GCF apoB concentration exhibited a good correlation with DM as well as the hyperlipidemic parameters.

It is now recognized that periodontal disease is a complication of DM [5,7]. The DM patients examined in this study showed significant increases in their periodontal scores as well as the DM parameters. To

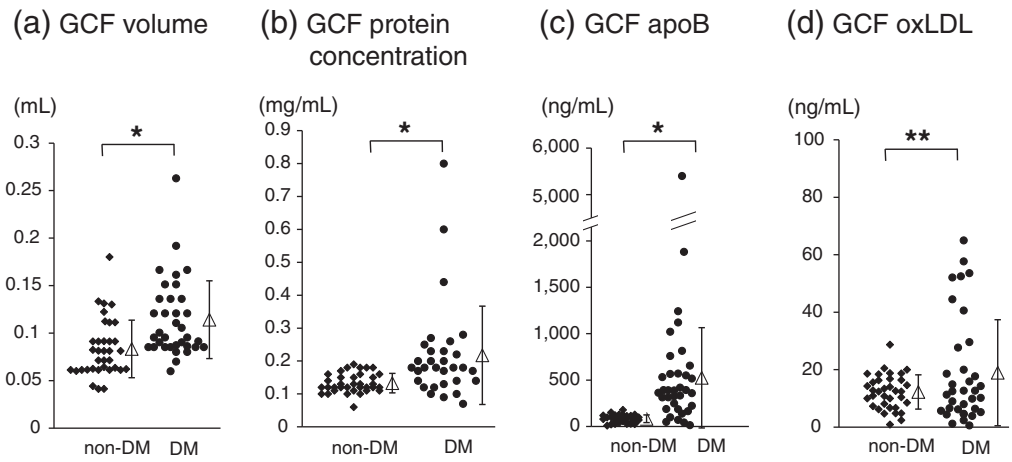

Fig. 1. The characteristics of the GCF samples from the DM patients and healthy subjects. The GCF samples were collected from two sites per subject. (a) GCF volume was measured using a pre-calibrated Periotron 8000. (b) The protein concentration in the GCF samples (recovered in 100 μL PBS) was measured using a BCA assay, then the original GCF protein concentration was estimated using the data on the GCF volume. The amounts of apoB (c) and oxLDL (d) in the GCF samples were determined by sandwich ELISA. Results are presented as the means ± S.D. Statistical analysis was performed using the Welch test. $^{*}p < 0.01$, $^{**}p < 0.05$.

minimize the possible effects of periodontitis on GCF, we collected GCF from non-diseased sites, since GCF from a diseased site could be a reflection of the inflammatory reactions in the gingiva as well as systemic conditions. In addition, although the volume of GCF increases when periodontal disease progresses, collecting GCF from diseased sites eroded by chronic inflammation is often accompanied by bleeding.

Our data show that the GCF volume and protein and oxLDL concentrations were significantly higher in the DM patients than in the healthy subjects. It cannot be denied that a local inflammatory response is also involved in the increase in GCF and the change of the components in GCF. We conjecture that the increased protein concentration and oxLDL level are not caused solely by local inflammatory responses, but rather, there must also be an effect of the systemic disease. The gingival capillaries are enriched in the tissue attached to teeth compared to the intraoral surface of the gingiva [18]. The volume and components of GCF may thus be strongly influenced by endothelial function in the capillaries. Some of pro-inflammatory cytokines, such as IL-1β and IL-8, are increased in the GCF from DM patients that increase vascular permeability [19,20]. It is well known that the vessels in many different tissues are damaged in DM patients, and increased extravasation of leukocytes and macromolecules under hyperglycemic conditions has been reported for gingival tissue [21].

ApoB and oxLDL in GCF showed no correlation with either apoB or oxLDL in plasma, suggesting that the permeation of apoB into GCF is independent of the plasma apoB concentration. Also, the oxLDL data confirms our previous report that the oxLDL/apoB ratio in GCF was 17-fold higher than that in plasma [15], supporting the notion that oxLDL in GCF does not simply originate from plasma. It is noteworthy that only 3 out of 18 DM patients in this study had history of diseases closely related to atherosclerosis, i.e. cardiovascular diseases, cerebral infarction, and atherosclerosis obliterans.

In addition, the apoB concentration in the DM patient GCF was 6-times higher than that in healthy subjects, while the GCF protein concentration in the DM patients was only 1.6-times higher than that in healthy subjects. No GCF sample from the healthy subjects had an apoB concentration higher than 250 ng/mL, while the plasma apoB concentration is estimated to be 5 μg/mL when it is diluted in PBS in the same way as GCF samples. It is thus suggested that the release of an extremely large protein-like apoB from plasma into GCF is tightly regulated. LDL can be transferred from the vessel lumen to the tissue area beyond the endothelial cell layer. Receptor-mediated transcytosis of LDL was demonstrated in endothelial cells from the aorta and brain capillaries in culture [22,23], and the in vivo evidence for the extravasation of LDL was also reported [24,25]. It is possible that LDL-receptor function in the gingival capillaries might be affected under DM conditions and thus the apoB concentration increases in GCF. This might also explain why the GCF apoB concentration correlates with the hyperlipidemic parameters in addition to the DM parameters. Further study is certainly needed to clarify the cause of the selective increase of apoB in GCF.

The ROC analysis of the GCF apoB concentration suggests that it may be a sensitive marker for hyperglycemic conditions, since the GCF apoB concentration is very stable in healthy people. GCF could be a clinical source to study some systemic conditions, not just the oral status. For example, if corroborated by further study, GCF may provide a way to contribute to a better cooperative treatment of patients with DM via both metabolic control and oral care.

The limitations of this study include the fact that the DM patients examined were under poor hyperglycemic control. We recruited the hospitalized patients enrolled in an education program so it would be possible to carefully examine the oral conditions of the DM patients during the stay in the hospital. Future study should examine a cohort with a larger number of patients with intermediate as well as severe hyperglycemia.

Table 4
Correlations between the GCF parameters and the hyperglycemia and hyperlipidemia parameters in plasma.

	HbA1c (NGSP)	GA	Fasting blood glucose	TG	LDL-C	MDA-LDL	ApoB	oxLDL
GCF-volume	.462*	.509*	.505*	.171	−.050	−.018	.123	−.153
GCF-protein	.183	.314**	.334**	−.031	−.042	.221	−.149	−.007
GCF-apoB	.333*	.398*	.459*	.319*	.290**	.473*	−.010	−.097
GCF-oxLDL	.265**	.303**	.209	.061	.289**	.476*	.128	−.157

* Correlations significant at $p < 0.01$ level by Pearson's correlation test.
** Correlations significant at $p < 0.05$ level by Pearson's correlation test.

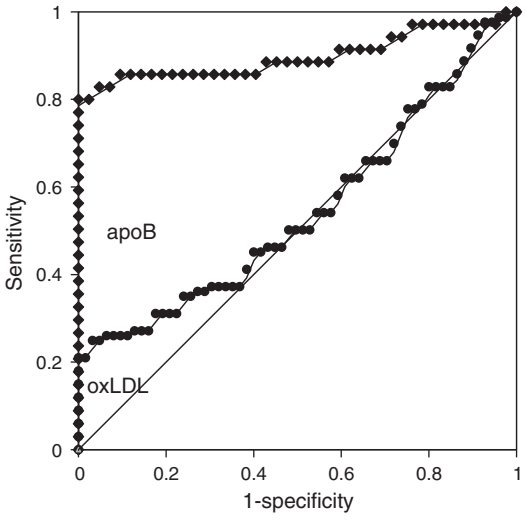

Fig. 2. Receiver operating characteristic (ROC) curves of the apoB and oxLDL concentrations in GCF for the prediction of DM. The ROC curves plot the sensitivity vs. 1-specificity by a gradual change in the cutoff value.

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